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(54) Title: A COLLAGEN BINDING PROTEIN AS WELL AS ITS PREPARATION

(57) Abstract

The present invention relates to a new recombinant hybrid-DNA-molecule comprising a nucleotide sequence from *S. aureus* coding for a protein, or polypeptide, having collagen binding properties.

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A COLLAGEN BINDING PROTEIN AS WELL AS ITS PREPARATION.DESCRIPTIONTechnical field

The present invention relates to a collagen
5 binding protein as well as hybrid-DNA-molecules, e.g.
plasmids or phages comprising a nucleotide sequence coding
for said protein. Further the invention relates to micro-
organisms comprising said molecules and their use producing
said protein, as well as the synthetic preparation of said
10 protein. In particular the invention relates to a cloned
gene encoding the Staphylococcus aureus collagen binding
protein, or functionally active portions thereof, vectors
containing the cloned gene or parts thereof, and micro-
organisms transformed by those vectors as well as the
15 cloning of the gene which specify the biosynthesis of
Staphylococcus aureus collagen binding protein (CBP) (also
called the collagen receptor by Switalski et al 1989) and
the use of organisms transformed with the cloned gene to
produce CBP or CBP like proteins. The invention also
20 describes the use of this gene for diagnostic purposes.

The object of the present invention is to obtain
a collagen binding protein.

A further object is to obtain said protein by
means of a genetic engineering technique by using e.g. a
25 plasmid comprising a nucleotide sequence coding for said
protein.

A further object is to obtain a possibility of
preparing said protein by chemical synthesis.

Further objects will be apparent from the
30 following description.

Background of the invention

WO-A1-85/05553 discloses bacterial cell surface
proteins having fibronectin, fibrinogen, collagen, and/or
laminin binding ability. Thereby it is shown that different
35 bacteria have an ability to bind to fibronectin, fibrino-
gen, collagen, and/or laminin.

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TACAAAACCA AAATTACGAA TGAACAGCAA AAAGAGTTTG TTAATAATTC
 ACAAGCTTGG TATCAAGAGC ATGGTAAGGA AGAGTGAAC GGGAAATCAT
 TTAATCATAC TGTGCACAAT ATTAATGCTA ATGCCGGTAT TGAAGGTACT
 GTAAAAGGTG AATTAAAAGT TTTAAAACAG GATAAAGATA CCAAGGCTCC
 5 TATAGCTAAT GTAAAATTTA AACTTTCTAA AAAAGATGGA TCAGTTGTAA
 AGGACAATCA AAAAGAAATT GAGATTATAA CAGATGCAAA CGGTATTGCT
 AATATTAAAG CGTTGCCTAG TGGAGACTAT ATTTTAAAAG AAATAGAGGC
 GCCACGACCG TATACATTTG ATAAGGATAA AGAATATCCG TTTACTATGA
 AAGATACAGA TAATCAGGGA TATTTTACGA CTATTGAAAA TGCAAAAGCG
 10 ATAGAAAAAA CAAAAGATGT TTCTGCTCAA AAGGTTTGGG AAGGCACTCA
 AAAAGTGAAA CCAACGATTT ATTTCAAGTT GTACAAACAA GATGACAATC
 AAAATACAAC ACCAGTAGAC AAAGCAGAGA TTAAAAAATT AGAAGATGGA
 ACGACAAAAG TGACATGGTC TAATCTTCCG GAAAATGACA AAAATGGCAA
 GGCTATTAAA TATTTAGTTA AAGAAGTAAA TGCTCAAGGT GAAGATACAA
 15 CACCAGAAGG ATATACTAAA AAAGAAAATG GTTTAGTGGT TACTAATACT
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 CAAAGACAAT CAAGATGGTA AGAGACCAGA AAAAGTCAGT GTGAATTTAT
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 TGGAAGTACG AATTTAAAGA CTTACCGAAG TATGATGAAG GAAAGAAAAT
 20 AGAATATACA GTGACCGAAG ATCACGTAAA AGACTACACA ACAGACATCA
 ACGGTACGAC AATAACGAAC AAGTATACAC CAGGAGAGAC ATCGGCAACA
 GTAACAAAAA ATTGGGATGA CAATAATAAC CAAGACGGAA AACGACCAAC
 TGAAATCAAA GTTGAGTTAT ATCAAGACGG AAAAGCAACA GGAAGAACGG
 CAACATTAAA TGAATCTAAT AACTGGACCC ATACGTGGAC AGGATTAGAT
 25 GAAAAAGCAA AAGGACAACA AGTAAAATAC ACAGTCGAGG AATTAACAAA
 GGTCAAAGGT TATACAACAC ATGTGGATAA CAATGATATG GGTAACCTGA
 TTGTGACGAA TAAATATACG CCAGAAACAA CATCAATTAG TGGTGAAAAA
 GTATGGGACG ACAAAGACAA TCAAGATGGT AAGAGACCAG AAAAAGTCAG
 TGTGAATTTA TTGGCTGATG GAGAGAAAGT AAAAACGTTA GACGTGACAT
 30 CTGAAACAAA CTGGAAGTAC GAATTTAAAG ACTTACCGAA GTATGATGAA
 GGAAAGAAAA TAGAATATAC AGTGACCGAA GATCACGTAA AAGACTACAC
 AACAGACATC AACGGTACGA CAATAACGAA CAAGTATACA CCAGGAGAGA
 CATCGGCAAC AGTAACAAAA AATTGGGATG ACAATAATAA CCAAGACGGA
 AAACGACCAA CTGAAATCAA AGTTGAGTTA TATCAAGACG GAAAAGCAAC
 35 AGGAAAAACG GCAACATTAA ATGAATCTAA TAACTGGACC CATACGTGGA
 CAGGATTAGA TGAAAAAGCA AAAGGACAAC AAGTAAAATA CACAGTCGAG

- 5 -

TyrGlySerTyrAsnSerPheSerIleAsnTyrLysThrLysIleThrAsnGluGlnGln
LysGluPheValAsnAsnSerGlnAlaTrpTyrGlnGluHisGlyLysGluGluValAsn
GlyLysSerPheAsnHisThrValHisAsnIleAsnAlaAsnAlaGlyIleGluGlyThr
ValLysGlyGluLeuLysValLeuLysGlnAspLysAspThrLysAlaProIleAlaAsn
5 ValLysPheLysLeuSerLysLysAspGlySerValValLysAspAsnGlnLysGluIle
GluIleIleThrAspAlaAsnGlyIleAlaAsnIleLysAlaLeuProSerGlyAspTyr
IleLeuLysGluIleGluAlaProArgProTyrThrPheAspLysAspLysGluTyrPro
PheThrMetLysAspThrAspAsnGlnGlyTyrPheThrThrIleGluAsnAlaLysAla
IleGluLysThrLysAspValSerAlaGlnLysValTrpGluGlyThrGlnLysValLys
10 ProThrIleTyrPheLysLeuTyrLysGlnAspAspAsnGlnAsnThrThrProValAsp
LysAlaGluIleLysLysLeuGluAspGlyThrThrLysValThrTrpSerAsnLeuPro
GluAsnAspLysAsnGlyLysAlaIleLysTyrLeuValLysGluValAsnAlaGlnGly
GluAspThrThrProGluGlyTyrThrLysLysGluAsnGlyLeuValValThrAsnThr
GluLysProIleGluThrThrSerIleSerGlyGluLysValTrpAspAspLysAspAsn
15 GlnAspGlyLysArgProGluLysValSerValAsnLeuLeuAlaAsnGlyGluLysVal
LysThrLeuAspValThrSerGluThrAsnTrpLysTyrGluPheLysAspLeuProLys
TyrAspGluGlyLysLysIleGluTyrThrValThrGluAspHisValLysAspTyrThr
ThrAspIleAsnGlyThrThrIleThrAsnLysTyrThrProGlyGluThrSerAlaThr
ValThrLysAsnTrpAspAspAsnAsnGlnAspGlyLysArgProThrGluIleLys
20 ValGluLeuTyrGlnAspGlyLysAlaThrGlyLysThrAlaThrLeuAsnGluSerAsn
AsnTrpThrHisThrTrpThrGlyLeuAspGluLysAlaLysGlyGlnGlnValLysTyr
ThrValGluGluLeuThrLysValLysGlyTyrThrThrHisValAspAsnAsnAspMet
GlyAsnLeuIleValThrAsnLysTyrThrProGluThrThrSerIleSerGlyGluLys
ValTrpAspAspLysAspAsnGlnAspGlyLysArgProGluLysValSerValAsnLeu
25 LeuAlaAspGlyGluLysValLysThrLeuAspValThrSerGluThrAsnTrpLysTyr
GluPheLysAspLeuProLysTyrAspGluGlyLysLysIleGluTyrThrValThrGlu
AspHisValLysAspTyrThrThrAspIleAsnGlyThrThrIleThrAsnLysTyrThr
ProGlyGluThrSerAlaThrValThrLysAsnTrpAspAspAsnAsnGlnAspGly
LysArgProThrGluIleLysValGluLeuTyrGlnAspGlyLysAlaThrGlyLysThr
30 AlaThrLeuAsnGluSerAsnAsnTrpThrHisThrTrpThrGlyLeuAspGluLysAla
LysGlyGlnGlnValLysTyrThrValGluGluLeuThrLysValLysGlyTyrThrThr
HisValAspAsnAsnAspMetGlyAsnLeuIleValThrAsnLysTyrThrProGluThr
ThrSerIleSerGlyGluLysValTrpAspAspLysAspAsnGlnAspGlyLysArgPro
GluLysValSerValAsnLeuLeuAlaAsnGlyGluLysValLysThrLeuAspValThr
35 SerGluThrAsnTrpLysTyrGluPheLysAspLeuProLysTyrAspGluGlyLysLys
IleGluTyrThrValThrGluAspHisValLysAspTyrThrThrAspIleAsnGlyThr

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The invention further comprises a microorganism containing at least one hybrid-DNA-molecule according to the above. The plasmid pSAC104 in an E. coli strain TG1 has been deposited at the Deutsche Sammlung von Mikroorganismen
5 (DSM), and has thereby been allocated the deposition number DSM 6199. The present invention provides a cloned gene encoding the CBP having improved CBP-properties as compared with native CBP which is released and purified from
10 *S. aureus* cells. The gene is derived from a *S. aureus* strain and inserted into a cloning vector. Cells of a procaryotic organism which have been transformed with recombinant vectors are disclosed.

The invention further provides the identification of the nucleotide sequence of the gene encoding the CBP
15 here called the cbp-gene. The deduced amino acid sequence reveals a molecule with several distinct features resembling staphylococcal cell surface proteins.

The invention also provides a procedure for production and purification of the recombinant CBP. This is
20 done in a way so that the molecule retains its collagen binding properties, thus this recombinant CBP resembles the native unreleased *S. aureus* CBP.

The invention further provides the use of the cbp-gene for diagnostic purposes. Gene probes chosen to
25 specifically recognize the presence of the cbp gene in clinical *S. aureus* isolates have been used. As an example, the correlation between the presence of CBP on the surface of *S. aureus* strains isolated from patient with septic arthritis could be verified by the presence of the cbp-gene
30 in all tested strains.

Appropriate carrier proteins can be coupled to the amino acid sequence as well, such as IgG binding regions of protein A.

The invention will be described in the following
35 with reference to the examples given, however, without being restricted thereto.

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S. aureus fibronectin receptor (●, ZZFR). Panel A - binding of ^{125}I -collagen to protein coated beads as a function of time. Panel B - inhibition of binding of ^{125}I -collagen by antibodies. Attachment of ^{125}I -labeled beads to cartilage as a function of time (panel C) and inhibition of attachment of ^{125}I -labeled beads to cartilage by antibodies (panel D). In this experiment 1 ug of adhesin protein was coupled to 10^8 polystyrene beads. Control beads were coated with the same molar concentration of the fibronectin receptor. Unreacted sites on the beads were saturated with bovine serum albumin. Scanning electron microscopy of beads coated with collagen adhesin (panel E) or fibronectin receptor protein (panel F) attached to cartilage.

Figure 6: Expression constructs utilized to localize the collagen binding domain within the S. aureus collagen adhesin.

Example 1:

20 Cloning and identification of the cbp-gene in E.coli

In order to isolate the gene encoding S. aureus CBP two commercial available (Clontech laboratories, Inc. Palo Alto, CA, USA) S. aureus strains (strain FDA 574 and FDA 485) were tested if they bound radioactivity labelled collagen. This was done according to Switalski et al 1989. Strain 574 was found to bind collagen and therefore a gene library (obtained from the same company, cat. #XL 15016) of the same strain was screened for the expression of CBP activity. Using the suppliers protocol (in addition to this protocol the general work involving molecular genetic appropriate protocols found in "Current Protocols in Molecular Biology" Vol. 1 and 2, (edited by Ausubel, F.M., R. Brent, R.E. Kingston, D.D. Moore, I.G. Seidman, J.A. Smith, U. Struhl, Greene, Wiley Interscience), and "Molecular Cloning". A laboratory manual, (Maniatis, T., Fritsch, E.F. and J. Sambrook (1982) Cold Spring Harbor

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to detect bound primary Fab-Fragments. After incubation for 1 h at RT the filters were washed 3 X 10 min in PBS-T. The bound labelled secondary antibodies were detected by a color reaction according to the manufacturer's instructions (Bio-Rad, Instructions for preparing the BCIP/NBT color development solution for use in the immun-blot alkaline phosphatase assay kit).

By the use the above described methods several recombinant lambda phages expressing CBP-activity could be identified and isolated.

Two of these were chosen for further studies. They were called lambda coll 1 and lambda cCOLR6A respectively.

Subcloning lambda coll 1: Purified lambda coll 1 DNA was cleaved with EcoRI and the sticky ends were filled in using Klenow fragments together with the dNTP's. The blunt ended DNA-fragments originating from the S. aureus chromosome were ligated into Sma 1 cleaved pUC 18 (Pharmacia-LKB Biotechnology, Uppsala, Sweden). After transformation into freeze competent E. coli TG1 cells recombinant clones were tested for expression of the CBP. It was found that all clones expressing CBP harboured a recombinant plasmid with an insert of approx. 4 kb. One such clone called p 16 was chosen for further studies and a schematic map of the insert in this clone is shown in Fig. 1 A.

In a similar way as lambda coll 1 two other lambda clones were generated from the screening of the genomic library. Large scale cultures of pure positives were obtained and the DNA was isolated. EcoRI digestion of the clones resulted in inserts with two different sizes. Clone 1A had an insert of 3.2 kb and 3B had an insert of 4.5 kb. The larger of the two was used for further characterization. Purified insert DNA (1.5 kb) from λ GT11 clone 3B was ligated to EcoRI digested puc18 and transformed into E. coli TB-1 cell creating subclone cCOLR6A. It was also subcloned into M13mp18/JM101 for sequencing.

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structure found in staphylococcal protein A (Guss et al 1984) and FnBP A (Signäs et al 1989) as well as streptococcal protein G (Guss et al 1986) and M protein (Hollingshead et al 1986). This region is thought to mediate the binding of the protein to the cell wall. The amino acid sequence nearest to the C-terminal end consists of a long stretch of hydrophobic residues followed by some charged amino acids. This region called M is similar in structure to the C-terminal end of protein A, FnBP A, Protein G and M protein.

The predicted mol.wt of the deduced CBP is approx. 133 kd (including the postulated signal sequence, S) which is very close to the mol.wt of 135 kd reported for the native released receptor (Switalski et al 1989).

In order to construct a plasmid coding for the complete cbp-gene *S. aureus* FDA 574 chromosomal DNA was purified and double cleaved with Hind III/Pst I. With the guidance of Southern Transfer experiments using a 32-P labelled oligonucleotide probe (5'-ATTAAAGCGTTGCCTAGTGG-3') it was known that cleavage with these enzymes should generate an approx. 3,2 kb fragment corresponding to the 3'end of the cbp-gene. After cleavage with these enzymes the chromosomal DNA was electrophoretically separated in an agarose gel. A gel slice roughly corresponding to right size was cut out and the DNA fragments eluted and purified. The purified fragments were ligated into pUC 18 previously double cleaved with Hind III/Pst I. After ligation followed transformation into *E. coli* TG1 and the resulting recombinant clones were screened for obtaining the right fragment using colony hybridization with the same probe. One positive clone hybridizing with the radioactive probe was chosen for further studies. This clone called *E. coli* pSAC 100 was cleaved with Hind III and a purified approx. 1,8 kb Hind III fragment from p 16 (encoding the 5'end of the cbp-gene, Fig. 1 A) was ligated into pSAC 100. After transformation

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activity. This result is in contrast with the findings reported by Switalski et al 1989 which found that purified or partly purified native collagen receptor could not inhibit the binding of collagen to S. aureus Cowan 1 cells. The
5 conclusion of this is that recombinant CBP expressed has retained more of its original features than the released protein from the staphylococci.

Although it was possible to detect CBP activity in the recombinant E. coli lysate it was not possible to
10 affinity purify the CBP using immobilized collagen or gelatine. Although in "Western transfer" experiments with lysates from the above mentioned recombinant clones, using the Fab-fragments described in Example 1, was it possible to detect bands corresponding to high mol.wt. fragments. These
15 were in the same size as expected from calculations using the deduced amino acid sequence.

Example 4:

Expression and of a CBP fusionprotein which retains the 20 collagen binding properties after purification

Been unsuccessful to affinity purify the recombinant produced CBP, using immobilized collagen, another approach was used. This approach was to fuse the cbp-gene or parts of the gene to another gene encoding a so called
25 affinity tail (Methods in enzymology, Part 185). The affinity tail to be tested was the part from the protein A gene encoding the IgG-binding domains (Uhle'n et al 1984). Therefore a vector encoding the above mentioned domains from protein A was used. This vector called pNSEQ1, which
30 was a gift from Dr. M. Uhle'n contains in addition to the IgG-binding domains (E, D, A, B and C) two multi cloning sites (MCS) which flank the IgG-binding domains. This makes it possible to chose a restriction enzyme that has a recognition site in both the MCS which upon cleavage results
35 in a release of (provided the restriction site is not present in the IgG-binding domains) a DNA fragment encoding the

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Example 5:The use of the CBP-gene as a diagnostic tool

Two oligonucleotides (JP-1, 5'-AGT-GGT-TAC-TAA-TAC-TG-3' and JP-2, 5'-CAG-GAT-AGA-TIG-GTT-TA-3') complementary to regions of the CBP that flanked the repeats B1, B2, and B3 were constructed (Oligo's Etc.). Genomic DNA from 6 different *Staphylococcus aureus* strains that were known to bind ^{125}I -collagen (Table 1) were isolated as previously described by Lindberg. Polymerase chain reaction (PCR) was performed with a Cetus/Perkin-Elmer DNA Thermocycler. Reaction mixtures (100 μl) contained 1mM of each primer, 200 mM of each dNTP, 1 mM Tris-HCl (pH 8.3), 5 mM KCl, 15 mM MgCl_2 , 0.001% gelatin, 3 μg template DNA, and 2.5 U AmpliTaq DNA polymerase. The reaction mixtures were overlaid with 100 μl of mineral oil and amplified for 30 cycles consisting of a 2 minute denaturation at 94°C, a 2 minute annealing period at 55°C, and a 3 minute extension period at 72°C. After amplification, 15 μl of the PCR products were analyzed on a 1% agarose gel (SeaKem GTG, FMC Inc., Rockland, Maine).

PCR analysis of the genomic DNA from the different *S. aureus* isolates revealed two distinctly different sized products. FDA 574, Cowan, and #13 all had gene products of 1677 bp, whereas Phillips, #7, and #14391 had gene products of 1118 bp. *S. aureus* Newman, a known non-collagen binder had no detectable PCR product. There is a direct correlation between the repeat size and the estimated molecular weight of the purified native collagen receptor from the different *S. aureus* strains tested. Upon further sequence analysis, it appears that a PCR product of 1677 bp corresponds to 3 repeat units, each 560 bp long. A PCR product of 1118 bp therefore corresponds to 2 repeats, each 560 bp long. These data correlate highly with the estimated molecular weight of purified native collagen receptors of 135 kd and 115 kd respectively.

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lysostaphin lysates prepared from different *S. aureus* isolates (Figure 3). Lysostaphin digestion releases from the cell surface of *S. aureus* a number of proteins, around 30 bands can be visualized in the lysates by Coomassie Brilliant Blue staining of the gel (Switalski et al., 1989). The anti-adhesin antibodies recognized a component of M_r 135 kd in the lysate of strain Cowan (Figure 3, lane a), which is in agreement with our previous observations (Switalski et al., 1989). The major immunoreactive protein detected in the lysates of the other collagen adhesin positive strains (CA+) varied in molecular weight and was present as either 110 kd or 135 kd (Figure 3, lanes b through h). No correlation was observed between the apparent size of the immunoreactive protein and the collagen binding capacity of a strain or its origin (bone, synovial fluid). None of the nine non-binding collagen *S. aureus* strains tested expressed an immunoreactive protein (Figure 3, lane i).

20 Collagen adhesin mediated attachment of staphylococci to collagenous substrata.

The relationship between the ability to express a collagen adhesin and the observed localization of an infection within collagen rich tissues prompted us to analyze the role of the cell surface adhesin in bacterial attachment to collagen containing substrates. We initially studied attachment of bacteria to surfaces coated with type II collagen. Results indicated that a collagen coated surface was an excellent attachment substrate for strains which express a surface localized collagen adhesin. The attachment is time dependent and saturable reaching an equilibrium after 3 hours of incubation (Figure 4A). The number of attaching bacteria is not influenced by the size of the adhesin since strains #14 and Phillips, which either express a 135 kd or 110 kd adhesin respectively, attached in equal numbers to the collagen coated substrate. When bacteria were preincubated with anti-adhesin antibodies, against the collagen

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Creation of artificial bacteria

"Artificial bacteria" were prepared by covalently coating polystyrene beads (1.2 μm vs. staphylococci 0.8 - 1.0 μm in diameter) with the collagen adhesin protein. These beads were then tested in a series of experiments analogous to those performed with intact bacteria. The collagen adhesin (CA) coated beads, but not beads coated with a recombinant form of another staphylococcal cell surface component, the fibronectin receptor (Flock et al., 1987), bound ^{125}I -collagen (Figure 5A) in a manner similar to that of CA+ strains of *S. aureus* (Speziale et al., 1986). This binding was abolished by anti-CA antibodies, whereas preimmune antibodies did not effectively inhibit binding (Figure 5B). When "artificial bacteria" were assayed for the ability to attach to collagen (data not shown) or cartilage, we found that CA beads adhered to the substrate in a time dependent manner, identical to that of CA+ strains of *S. aureus*, while beads coated with the fibronectin receptor did not adhere at significant levels (Figure 5C). The anti-CA antibody inhibited the adhesion of CA beads to cartilage in a dose dependent fashion, whereas a preimmune antibodies had no effect (Figure 5D). Once again the quantitative binding data was corroborated by electron microscopy observations. CA coated beads attached in large numbers to cartilage tissue, in particular to collagen fibers (Figure 5E), while beads coated with the fibronectin receptor did not (Figure 5F).

Localization of the collagen binding domain within the collagen adhesin.

Various expression constructs have been created in *E. coli* in effort to specifically localize the collagen binding domain. Two different types of expression vectors have been utilized in these experiments, pKK223-3 and pGEX-2T, the second of which results in the collagen adhesin fused to glutathione-S-transferase. To date the smallest

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including man, the protein, or polypeptide is dispersed in sterile, isotonic saline solution, optionally while adding a pharmaceutically acceptable dispersing agent. Different types of adjuvants can further be used in order to sustain the release in the tissue, and thus expose the protein or the peptide for a longer time to the immunodefense system of a body.

A suitable dosage to obtain immunization is 0,5 to 5 μg of CBP, or polypeptide, per kg bodyweight and injection of immunization. In order to obtain a durable immunization, vaccination should be carried out at more than one consecutive occasion with an interval of 1 to 3 weeks, preferably at three occasions.

When using the present CBP, or polypeptide, for topical, local administration the protein is dispersed in an isotonic saline solution to a concentration of 25 to 250 μg per ml. The wounds are then treated with such an amount only to obtain a complete wetting of the wound surface. For an average wound thus only a couple of millilitres of solution are used in this way. After treatment using the protein solution the wounds are suitably washed with isotonic saline or another suitable wound treatment solution.

Further the collagen binding protein as well as the minimal collagen binding site polypeptide, of the present invention can be used to diagnose bacterial infections caused by Staphylococci strains, whereby a collagen binding protein of the present invention is immobilized on a solid carrier, such as small latex or Sepharose^R beads, whereupon sera containing antibodies are allowed to pass and react with the CBP thus immobilized. The agglutination is then measured by known methods.

Further, the CBP, or the polypeptide can be used in an ELISA test (Enzyme Linked Immuno Sorbent Assay; E Engvall, Med. Biol. 55, 193, (1977)). Hereby wells in a polystyrene microtitre plate are coated with the CBP, and incubated over night at 4°C. The plates are then thorough-

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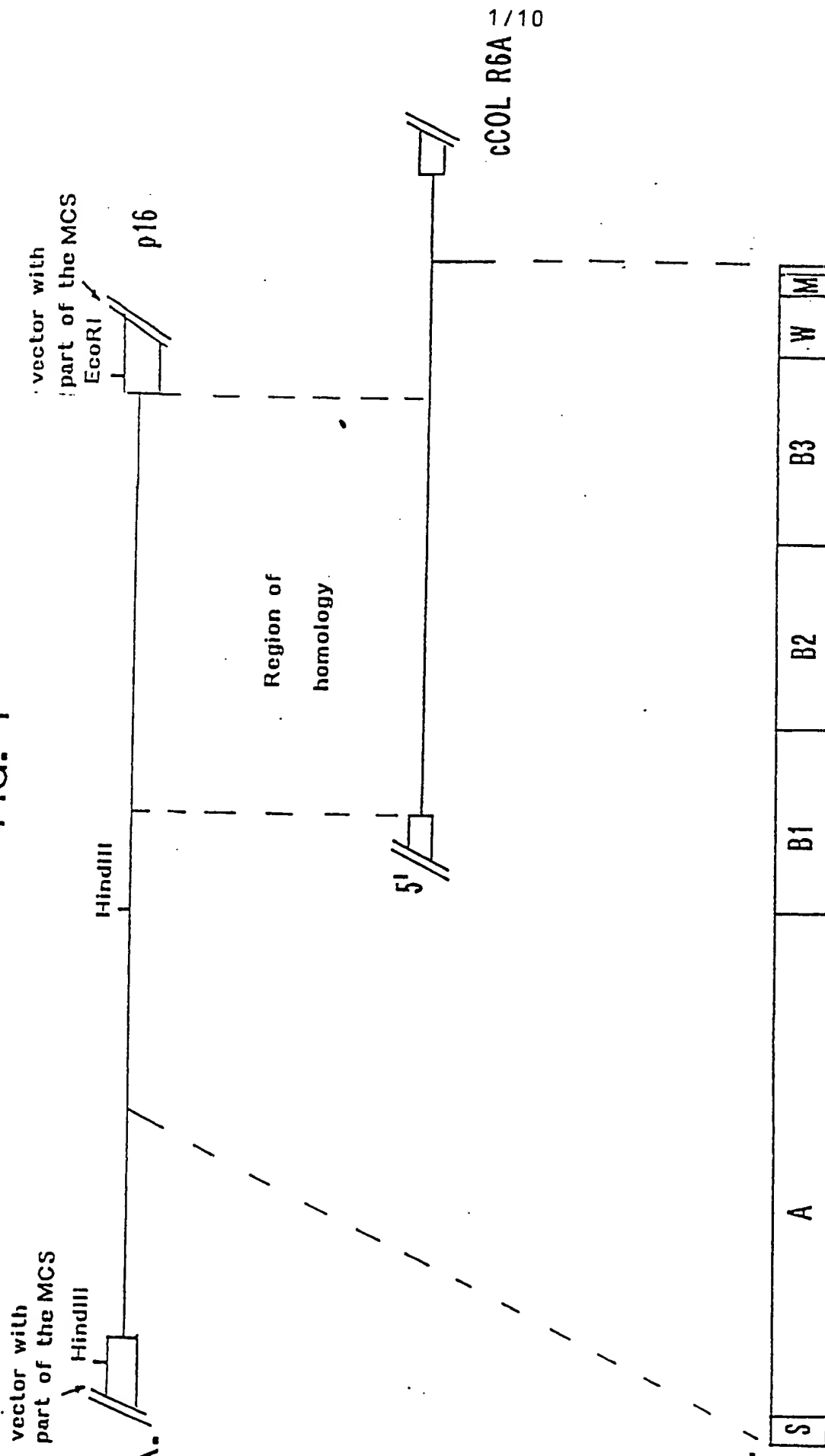
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 AAAATACAAC ACCAGTAGAC AAAGCAGAGA TTAAAAAATT AGAAGATGGA
 ACGACAAAAG TGACATGGTC TAATCTTCCG GAAAATGACA AAAATGGCAA
 GGCTATTAAA TATTTAGTTA AAGAAGTAAA TGCTCAAGGT GAAGATACAA
 15 CACCAGAAGG ATATACTAAA AAAGAAAATG GTTTAGTGGT TACTAATACT
 GAAAAACCAA TCGAAACAAC ATCAATTAGT GGTGAAAAAG TATGGGACGA
 CAAAGACAAT CAAGATGGTA AGAGACCAGA AAAAGTCAGT GTGAATTTAT
 TGGCTAACGG GGAGAAAGTA AAAACGTTAG ACGTGACATC TGAAACAAAC
 TGGAAGTACG AATTTAAAGA CTTACCGAAG TATGATGAAG GAAAGAAAAT
 20 AGAATATACA GTGACCGAAG ATCACGTAAA AGACTACACA ACAGACATCA
 ACGGTACGAC AATAACGAAC AAGTATACAC CAGGAGAGAC ATCGGCAACA
 GTAACAAAAA ATTGGGATGA CAATAATAAC CAAGACGGAA AACGACCAAC
 TGAAATCAAA GTTGAGTTAT ATCAAGACGG AAAAGCAACA GGAAAAACGG
 CAACATTAAA TGAATCTAAT AACTGGACCC ATACGTGGAC AGGATTAGAT
 25 GAAAAAGCA AGGACAACA AGTAA ATAC ACAGTCGAGG AATTAACAAA
 GGTCAAAGGT ATACAACAC ATGTGGATAA CAATGATATG GGTAACTTGA
 TTGTGACGAA TAAATATACG CCAGAAACAA CATCAATTAG TGGTGAAAAA
 GTATGGGACG ACAAAGACAA TCAAGATGGT AAGAGACCAG AAAAAGTCAG
 TGTGAATTTA TTGGCTGATG GAGAGAAAGT AAAAACGTTA GACGTGACAT
 30 CTGAAACAAA CTGGAAGTAC GAATTTAAAG ACTTACCGAA GTATGATGAA
 GGAAAGAAAA TAGAATATAC AGTGACCGAA GATCACGTAA AAGACTACAC
 AACAGACATC AACGGTACGA CAATAACGAA CAAGTATACA CCAGGAGAGA
 CATCGGCAAC AGTAACAAAA AATTGGGATG ACAATAATAA CCAAGACGGA
 AAACGACCAA CTGAAATCAA AGTTGAGTTA TATCAAGACG GAAAAGCAAC
 35 AGGAAAAACG GCAACATTAA ATGAATCTAA TAACTGGACC CATACGTGGA
 CAGGATTAGA TGAAAAAGCA AAAGGACAAC AAGTAAAATA CACAGTCGAG

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10. Chemical synthesis method for producing a collagen binding protein or polypeptide according to claim 1, whereby an amino acid residue is built up based on said nucleotide sequence encoding for said protein or polypeptide starting
 5 from the C-terminal serine, which is stepwise reacted with the appropriate amino acid, whereby it is finally reacted with alanine at the N-terminal end, to form the collagen binding protein or polypeptide.

10 11. A collagen binding protein or polypeptide comprising at least one of the amino acid sequence
 Ala
 ArgAspIleSerSerThrAsnValThrAspLeuThrValSerProSerLysIleGluAsp
 GlyGlyLysThrThrValLysMetThrPheAspAspLysAsnGlyLysIleGlnAsnGly
 15 AspMetIleLysValAlaTrpProThrSerGlyThrValLysIleGluGlyTyrSerLys
 ThrValProLeuThrValLysGlyGluGlnValGlyGlnAlaValIleThrProAspGly
 AlaThrIleThrPheAsnAspLysValGluLysLeuSerAspValSerGlyPheAlaGlu
 PheGluValGlnGlyArgAsnLeuThrGlnThrAsnThrLeuAspAspLysValAlaThr
 IleThrSerGlyAsnLysSerThrAsnValIleGlyTrpIleLysValLysArgGluPro
 20 ValValPheLeuIleAsnLysSerGlyLysIleCysTyrGlnGluAspThrThrHisVal
 ArgTrpPheLeuAsnIleAsnAsnGluLysSerTyrValSerLysAspIleThrIleLys
 AspGlnIleGlnGlyGlyGlnGlnLeuAspLeuSerThrLeuAsnIleAsnValThrGly
 ThrHisSerAsnTyrTyrSerGlyGlnSerAlaIleThrAspPheGluLysAlaPhePro
 GlySerLysIleThrValAspAsnThrLysAsnThrIleAspValThrIleProGlnGly
 25 TyrGlySerTyrAsnSerPheSerIleAsnTyrLysThrLysIleThrAsnGluGlnGln
 LysGluPheValAsnAsnSerGlnAlaTrpTyrGlnGluHisGlyLysGluGluValAsn
 GlyLysSerPheAsnHisThrValHisAsnIleAsnAlaAsnAlaGlyIleGluGlyThr
 ValLysGlyGluLeuLysValLeuLysGlnAspLysAspThrLysAlaProIleAlaAsn
 ValLysPheLysLeuSerLysLysAspGlySerValValLysAspAsnGlnLysGluIle
 30 GluIleIleThrAspAlaAsnGlyIleAlaAsnIleLysAlaLeuProSerGlyAspTyr
 IleLeuLysGluIleGluAlaProArgProTyrThrPheAspLysAspLysGluTyrPro
 PheThrMetLysAspThrAspAsnGlnGlyTyrPheThrThrIleGluAsnAlaLysAla
 IleGluLysThrLysAspValSerAlaGlnLysValTrpGluGlyThrGlnLysValLys
 ProThrIleTyrPheLysLeuTyrLysGlnAspAspAsnGlnAsnThrThrProValAsp
 35 LysAlaGluIleLysLysLeuGluAspGlyThrThrLysValThrTrpSerAsnLeuPro
 GluAsnAspLysAsnGlyLysAlaIleLysTyrLeuValLysGluValAsnAlaGlnGly

FIG. 1



1141 GACATGATTAAAGTGGCATGGCCGACAAGCGGTACAGTAAAGATAGAGGGTTATAGTAAA
 AspMetIleLysValAlaTrpProThrSerGlyThrValLysIleGluGlyTyrSerLys 1200
 1201 ACAGTACCATTAACTGTTAAAGGTGAACAGGTGGGTCAAGCAGTTATTACACCAGACGGT
 ThrValProLeuThrValLysGlyGluGlnValGlyGlnAlaValIleThrProAspGly 1260
 1261 GCAACAATTACATTCAATGATAAAGTAGAAAAATTAAGTGATGTTTCGGGATTTCAGAA
 AlaThrIleThrPheAsnAspLysValGluLysLeuSerAspValSerGlyPheAlaGlu 1320
 1321 TTTGAAGTACAAGGAAGAAATTTAAGCACAACAAATACCTTAGATGACAAAGTAGCTACG
 PheGluValGlnGlyArgAsnLeuThrGlnThrAsnThrLeuAspAspLysValAlaThr 1380
 1381 ATAACATCTGGGAATAAATCAACGAATGTTATCGGTGGATAAAGTGAAGCGGGAACCA
 IleThrSerGlyAsnLysSerThrAsnValIleGlyTrpIleLysValLysArgGluPro 1440
 1441 GTAGTGTCTTCTAATTAATAAAGCGGGAAGATATGCTACCAAGAAGATACGACACATGTA
 ValValPheLeuIleAsnLysSerGlyLysIleCysTyrGlnGluAspThrThrHisVal 1500
 1501 CGATGGTTTTTAAATATTAAACAATGAAAAAGTTATGTATCGAAAGATATTACTATAAAG
 ArgTrpPheLeuAsnIleAsnAsnGluLysSerTyrValSerLysAspIleThrIleLys 1560
 1561 GATCAGATTCAAGGTGGACAGCAGTTAGATTTAAGCACATTAAACATTATGTGACAGGT
 AspGlnIleGlnGlyGlyGlnGlnLeuAspLeuSerThrLeuAsnIleAsnValThrGly 1620
 1621 ACACATAGCAATTATTATAGTGGACAAAGTGCAATTACTGATTTTGAAAAAGCCTTTCCA
 ThrHisSerAsnTyrTyrSerGlyGlnSerAlaIleThrAspPheGluLysAlaPhePro 1680
 1681 GGTCTCTAAAATAACTGTTGATAATACGAAGAACACAATTGATGTAACAATTCCACAAGGC
 GlySerLysIleThrValAspAsnThrLysAsnThrIleAspValThrIleProGlnGly 1740
 1741 TATGGGTCATATAATAGTTTTTCAATTAACACAAAACCAAATTACGAATGAACAGCAA
 TyrGlySerTyrAsnSerPheSerIleAsnTyrLysThrLysIleThrAsnGluGlnGln
 HindIII
 1801 AAAGAGTTTGTAAATAATTCACAAAGCTTGGTATCAAGAGCATGGTAAGGAAGAAGTGAAC
 LysGluPheValAsnAsnSerGlnAlaTrpTyrGlnGluHisGlyLysGluGluValAsn 1860
 1861 GGGAAATCATTTAATCATACTGTGCACAATATTAATGCTAATGCCGGTATTGAAGGTACT
 GlyLysSerPheAsnHisThrValHisAsnIleAsnAlaAsnAlaGlyIleGluGlyThr 1920
 1921 GTAAAAGGTGAATTAAGTTTAAACAGGATAAAGATACCAAGGCTCCTATAGCTAAT
 ValLysGlyGluLeuLysValLeuLysGlnAspLysAspThrLysAlaProIleAlaAsn 1980
 1981 GTAAAATTTAACTTTCTAAAAAGATGGATCAGTTGTAAAGGACAATCAAAAAGAAATT
 ValLysPheLysLeuSerLysLysAspGlySerValValLysAspAsnGlnLysGluIle 2040
 2041 GAGATTATAACAGATGCAACCGTATTGCTAATATTAAGCGTTGCCTAGTGGAGACTAT
 GluIleIleThrAspAlaAsnGlyIleAlaAsnIleLysAlaLeuProSerGlyAspTyr 2100
 ATTTTAAAGAAATAGAGGCCACGACCGTATACATTTGATAAGGATAAAGAAATTCG

FIG. 2

- (cont'd)

3121 GTATGGGACGACAAAGACAATCAAGATGGTAAGAGACCAGAAAAAGTCAGTGTGAATTTA
 -----+-----+-----+-----+-----+-----+-----+-----+
 ValTrpAspAspLysAspAsnGlnAspGlyLysArgProGluLysValSerValAsnLeu 3180

 3181 TTGGCTGATGGAGAGAAAGTAAAAACGTTAGACGTGACATCTGAAACAAACTGGAAGTAC
 -----+-----+-----+-----+-----+-----+-----+-----+ 3240
 LeuAlaAspGlyGluLysValLysThrLeuAspValThrSerGluThrAsnTrpLysTyr

 3241 GAATTTAAAGACTTACCGAAGTATGATGAAGGAAAGAAATAGAATATACAGTGACCGAA
 -----+-----+-----+-----+-----+-----+-----+-----+ 3300
 GluPheLysAspLeuProLysTyrAspGluGlyLysLysIleGluTyrThrValThrGlu

 3301 GATCACGTAAAAGACTACACAACAGACATCAACGGTACGACAATAACGAACAAGTATACA
 -----+-----+-----+-----+-----+-----+-----+-----+ 3360
 AspHisValLysAspTyrThrThrAspIleAsnGlyThrThrIleThrAsnLysTyrThr

 3361 CCAGGAGAGACATCGGCAACAGTAACAAAAAATTGGGATGACAATAATAACCAAGACGGA
 -----+-----+-----+-----+-----+-----+-----+-----+ 3420
 ProGlyGluThrSerAlaThrValThrLysAsnTrpAspAspAsnAsnGlnAspGly

 3421 AAACGACCAACTGAAATCAAAGTTGAGTTATATCAAGACGGAAAAAGCAACAGGAAAAACG
 -----+-----+-----+-----+-----+-----+-----+-----+ 3480
 LysArgProThrGluIleLysValGluLeuTyrGlnAspGlyLysAlaThrGlyLysThr

 3481 GCAACATTAAATGAATCTAATAACTGGACCCATACGTGGACAGGATTAGATGAAAAAGCA
 -----+-----+-----+-----+-----+-----+-----+-----+ 3540
 AlaThrLeuAsnGluSerAsnAsnTrpThrHisThrTrpThrGlyLeuAspGluLysAla

 3541 AAAGGACAACAAGTAAAATACACAGTCGAGGAATTAACAAAGGTCAAAGGTTATACAACA
 -----+-----+-----+-----+-----+-----+-----+-----+ 3600
 LysGlyGlnGlnValLysTyrThrValGluGluLeuThrLysValLysGlyTyrThrThr
 CATGTGGATAACAATGATATGGGCAACTTGATTGTGACGAATAAATATACGCCAGAAACA → B3
 -----+-----+-----+-----+-----+-----+-----+-----+ 3660
 HisValAspAsnAsnAspMetGlyAsnLeuIleValThrAsnLysTyrThrProGluThr

 3661 ACATCAATTAGCGGTGAAAAAGTATGGGACGACAAAGACAATCAAGATGGTAAGAGACCA
 -----+-----+-----+-----+-----+-----+-----+-----+ 3720
 ThrSerIleSerGlyGluLysValTrpAspAspLysAspAsnGlnAspGlyLysArgPro

 3721 GAAAAAGTCAGTGTAAATTTATTGGCTAACGGAGAGAAAGTAAAAACGTTAGACGTGACA
 -----+-----+-----+-----+-----+-----+-----+-----+ 3780
 GluLysValSerValAsnLeuLeuAlaAsnGlyGluLysValLysThrLeuAspValThr

 3781 TCTGAAACAAACTGGAAGTACGAATTTAAAGACTTACCGAAGTATGATGAAGGAAAGAAA
 -----+-----+-----+-----+-----+-----+-----+-----+ 3840
 SerGluThrAsnTrpLysTyrGluPheLysAspLeuProLysTyrAspGluGlyLysLys

 3841 ATAGAATATACAGTGACCGAAGATCACGTAAAAGACTACACAACAGACATCAACGGTACG
 -----+-----+-----+-----+-----+-----+-----+-----+ 3900
 IleGluTyrThrValThrGluAspHisValLysAspTyrThrThrAspIleAsnGlyThr
 3' end of insert in p16 ←
 3901 ACAATAACGAACAAGTATACACCAGGAGAGACATCGGCAACAGTAACAAAAAATTGGGAT
 -----+-----+-----+-----+-----+-----+-----+-----+ 3960
 ThrIleThrAsnLysTyrThrProGlyGluThrSerAlaThrValThrLysAsnTrpAsp

 3961 GACAATAATAACCAAGACGGAAAACGACCAACTGAAATCAAAGTTGAGTTATATCAAGAT
 -----+-----+-----+-----+-----+-----+-----+-----+ 4020

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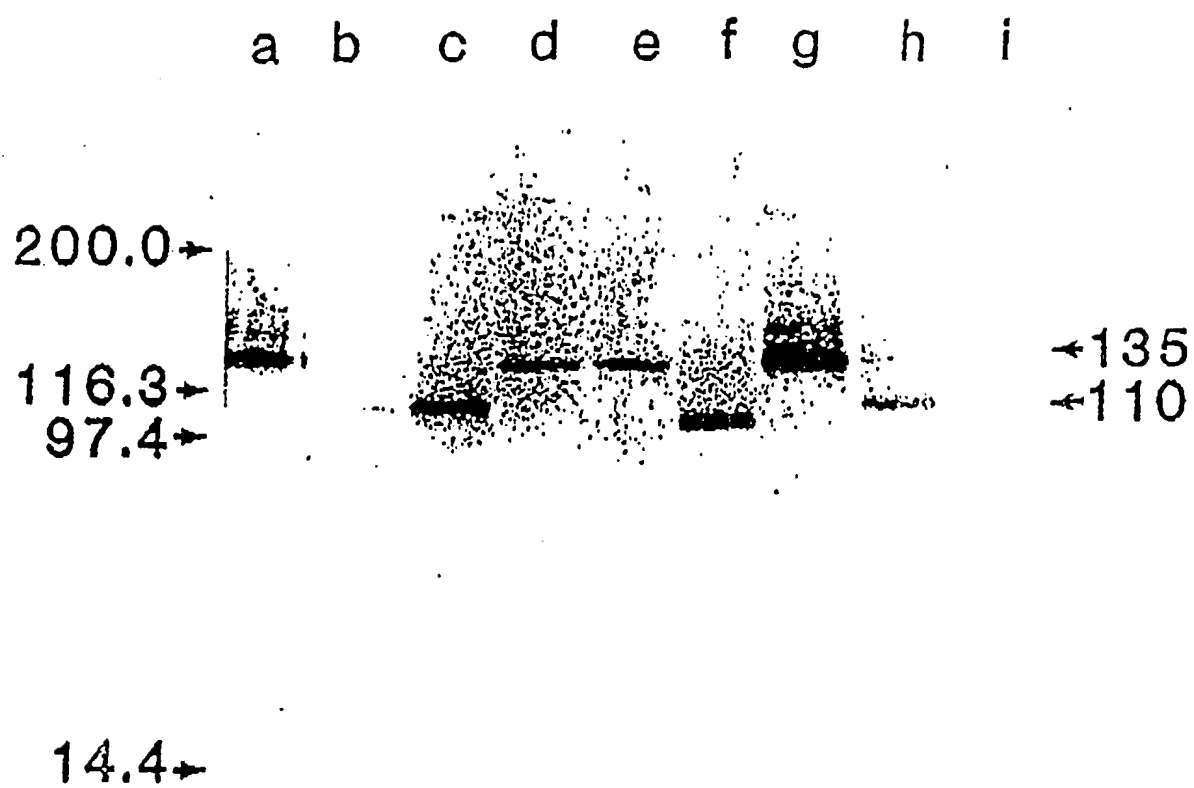


FIG. 3

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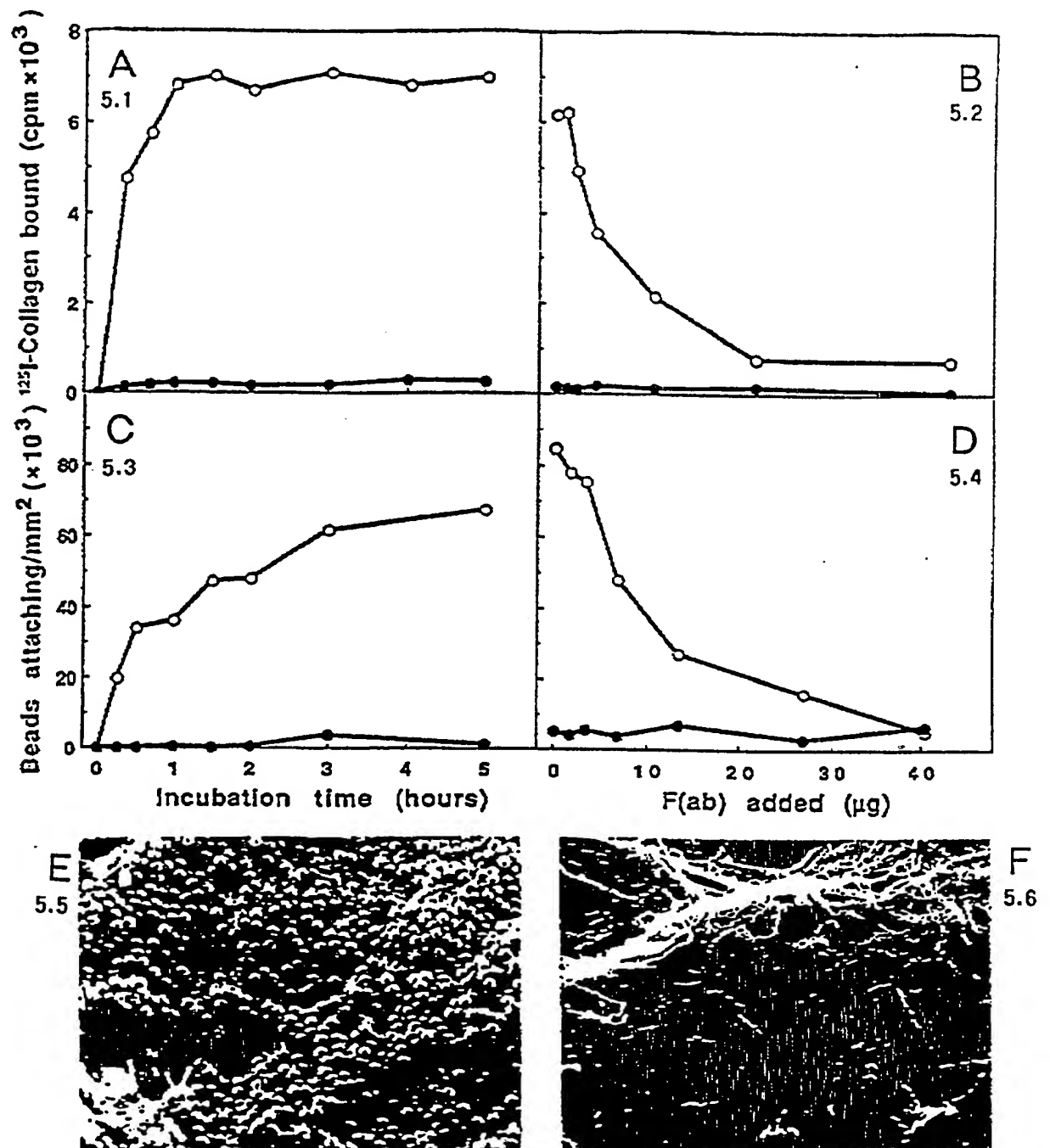
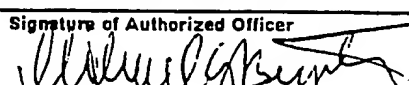


FIG. 5

INTERNATIONAL SEARCH REPORT

International Application No PCT/SE 91/00707

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) ⁶ According to International Patent Classification (IPC) or to both National Classification and IPC IPC5: C 07 K 15/04		
II. FIELDS SEARCHED		
Minimum Documentation Searched ⁷		
Classification System	Classification Symbols	
IPC5	C 07 K; C 12 N	
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in Fields Searched ⁸		
SE,DK,FI,NO classes as above		
III. DOCUMENTS CONSIDERED TO BE RELEVANT⁹		
Category *	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³
X	EP, A2, 0163623 (ALFA-LAVAL AGRI INTERNATIONAL AB) 4 December 1985, see page 8 lines 7-15 --	1-11
X	Dialog Information Services, file 55: BIOSIS 85-92, Dialog accession no. 7397648, Switalski L M et al: "Isolation and characterization of a putative collagen receptor from staphylococcus- aureus strain cowan 1", & J Biol Chem 264 (35) 1989 21080-21086, -- -----	1-11
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IV. CERTIFICATION		
Date of the Actual Completion of the International Search		Date of Mailing of this International Search Report
3rd February 1992		1992 -02- 06
International Searching Authority		Signature of Authorized Officer
		

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